Stereochemistry of Conversion of the Suicide Substrates β-Chloro-D-alanine and D- and L-Serine *O*-Sulfates into Pyruvate by D-Amino Acid Aminotransferase and by L-Aspartate Aminotransferase

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β-Chloro-D-alanine and D-serine *O*-sulfate are converted into a putative aminoacrylate intermediate by D-amino acid aminotransferase. This either reacts with pyridoxal phosphate to form a reactive inhibitor of the enzyme or it is protonated and hydrolysed to give pyruvate. The protonation reaction is shown to occur with modest stereoselectivity, indicating overall retention of stereochemistry in replacement of the leaving group by hydrogen. The corresponding reaction of L-serine *O*-sulfate using L-aspartate aminotransferase shows little or no stereoselectivity.

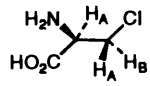
β-Chloroalanine **1** and serine *O*-sulfate **2** (R = SO_3H) act as suicide substrates for a variety of pyridoxal phosphate (PLP)-dependent enzymes. It has been found that inhibition normally competes with turnover to yield pyruvate, ammonia and either chloride or sulfate ion. For β-chloroalanine, for example, the ratio of turnover to inhibition has been shown to be ~1500:1 with the enzyme D-amino acid aminotransferase;[1] ~850:1 with the enzymes amino acid racemase[2] and alanine racemase;[3,4] ~300:1 for mitochondrial aspartate aminotransferase;[5] and ~100:1 for cytosolic aspartate aminotransferase.[5]

It was originally suggested[1] that the mechanism for these reactions was as shown in Scheme 1 with primary formation of the aldimine **4** with PLP **3** followed by tautomerism to the quinone form **5** and elimination to the intermediate Michael acceptor **6**. Inhibition would result from attack of a nucleophile at the active site to yield the covalent adduct **7**, whereas hydrolysis of the intermediate **6** would result in formation of pyruvate **8**.

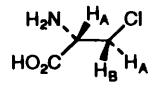
Doubts were cast on this mechanism by the discovery that basic treatment of the products of inhibition of cytosolic aspartate aminotransferase by β-chloroalanine[6] or serine *O*-sulfate[7] or of glutamate decarboxylase with serine *O*-sulfate[8] gave a product **13** previously noted by Schnackerz *et al.*[9] as an intermediate in a reaction catalysed by D-serine dehydratase. Identical findings for inhibition of alanine racemase with β-chloro-D- and L-alanine[2,4] supported a mechanism suggested by Metzler[8] and shown in Scheme 2. By this mechanism, inhibition occurs by reaction of the aldimine moiety of the enamine **6** with an active-site lysine residue to give the adduct **9**. This then allows elimination of aminoacrylate **11** which will then react as an enamine with the imine **10** with carbon–carbon bond formation to give the covalent adduct **12**. Treatment of this adduct with base then yields the so-called 'Schnackerz' compound **13**. By this mechanism, pyruvate would arise by protonation of the acrylate **11** and hydrolysis of the resultant iminium species **14** as shown in Scheme 3.

Results and Discussion

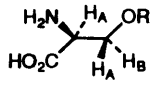
As part of a general study of the stereochemistry of inhibition of enzymes catalysing the reactions of D- and L-amino acids we have synthesized (2*S*,3*S*)-β-chloro-[3-$^2H_1$]- and (2*S*,3*R*)-β-chloro-[2,3-$^2H_2$] alanine, **1b** (H_B = ^2H) and **1b** (H_A = ^2H), respectively.[10] We had also prepared (2*S*,3*R*)-[3-$^2H_1$]- and (2*S*,3*S*)-[2,3-$^2H_2$]-serine, **2a** (R = H, H_B = ^2H) and **2a** (R = H, H_A = ^2H), respectively[11] and (2*R*,3*S*)-[3-$^2H_1$]- and



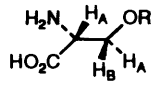
1a



1b



2a

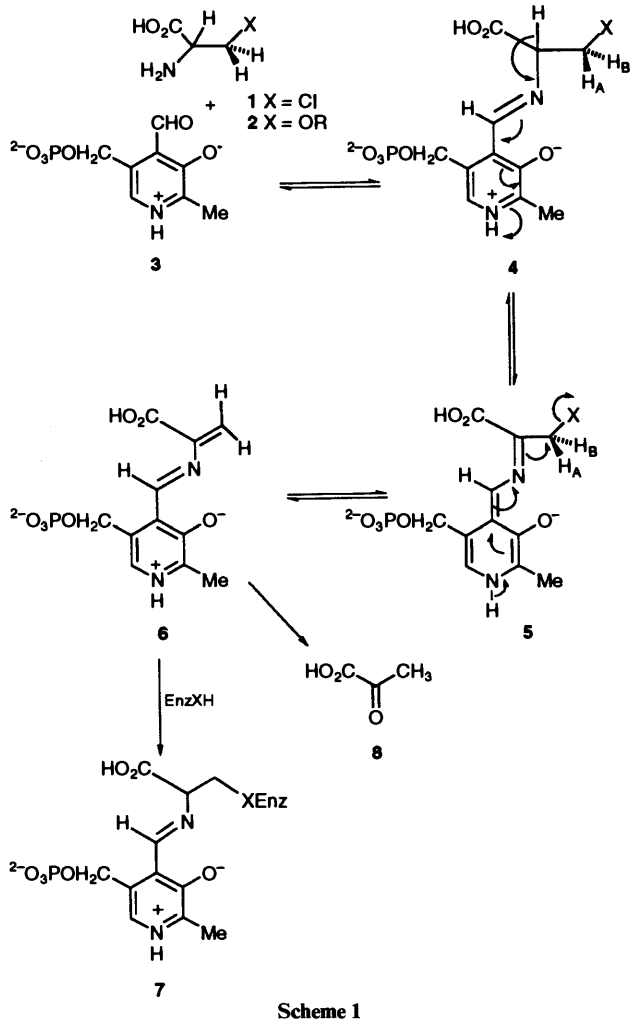


2b

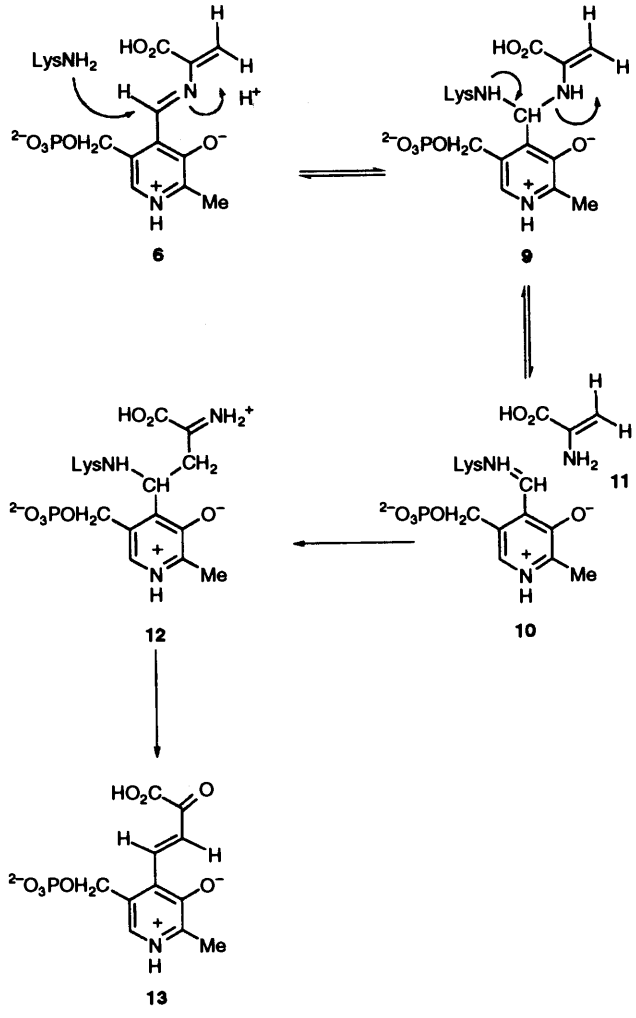
(2*R*,3*R*)-[2,3-$^2H_2$]-serine **2b** (R = H, H_B = ^2H) and **2b** (R = H, H_A = ^2H), respectively[10] and have converted these into the corresponding labelled samples of L- and D-serine *O*-sulfate **2a** and **2b** (R = SO_3H) by using the method developed by Previero *et al.*[12] We were therefore in a position to study the stereochemistry of the turnover of these inhibitors to pyruvic acid by enzymes which separately and specifically catalyse reactions of L- and D-amino acids, especially in the light of the finding[13] that D-amino acid aminotransferase and one other aminotransferase have different stereospecificities for C-4′ transfer than have L-aspartate aminotransferase, L-alanine aminotransferase and all other aminotransferases. It is also of interest that L- and D-serine-*O*-sulfates have been shown to inhibit L-glutamate decarboxylase by different mechanisms.[14]

D-Amino acid aminotransferase (EC 2.6.1.21) was therefore isolated from *Bacillus sphaericus*† and purified by combination and adaptation of the methods of Soda *et al.*[15] and Jones *et al.*[16] We then compared the efficiency of this enzyme at converting β-chloro-D-alanine **1b**, and D-serine *O*-sulfate **2b** (R = SO_3H) into pyruvate **8** by incubating these substrates separately with the enzyme in the presence of lactate dehydrogenase (EC 1.1.1.27) and the coenzyme 1,4-dihydronicotinamide adenine dinucleotide (NADH). As pyruvate was produced, it was immediately converted into L-lactate **15** by the dehydrogenase, NADH being converted into NAD^+ in the process (see Scheme 4). The decrease in absorption at 340 nm in the UV spectrum was used to measure the conversion of substrate into pyruvate and the results are plotted in Fig. 1. Curve (a) shows the results for β-chloro-D-alanine as substrate and curve (b) for D-serine *O*-

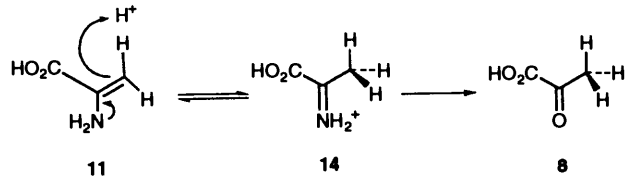
† Grown by Dr. R. Eady and his colleagues, Nitrogen Fixation Laboratory, University of Sussex.



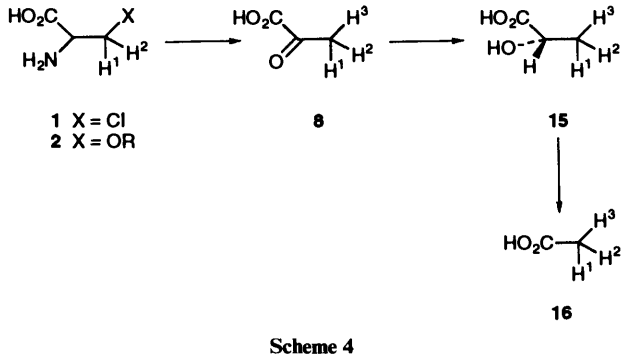
Scheme 1



Scheme 2



Scheme 3



Scheme 4

sulfate as substrate. It is evident that β-chloro-D-alanine is the better substrate and the diminution in the rate of pyruvate production with time was taken to be an indication of the competing inhibition reaction of the substrates.

Similar studies were conducted using L-serine *O*-sulfate and the commercially obtained enzymes L-aspartate aminotransferase (EC 2.6.1.1) and L-alanine aminotransferase (EC 2.6.1.2). The results are shown as curves (c) and (d) in Fig. 1. L-Serine *O*-sulfate was evidently not a substrate for L-alanine aminotransferase, although it was a substrate for L-aspartate aminotransferase. L-Alanine aminotransferase was shown to convert its natural substrate, L-alanine, efficiently into pyruvate in the presence of α-ketoglutarate (2-oxopentanedioic acid) when using the lactate dehydrogenase/NADH system [Fig. 2, curve (a)]. However, addition of L-serine *O*-sulfate after 5 min [Fig. 2, curve (b)] caused the rate of consumption of NADH to be diminished considerably, indicating inhibition of the enzyme by L-serine *O*-sulfate. When L-serine *O*-sulfate was incubated with the enzyme and α-ketoglutarate and L-alanine was added after 5 min [Fig. 2, curve (c)] then consumption of NADH commenced at this time at approximately the same diminished rate as had been seen in Fig. 2, curve (b) after addition of L-serine *O*-sulfate to the incubation of L-alanine with L-alanine aminotransferase. Comparable amounts of substrate, inhibitor and enzyme were used in all experiments.

We were now in a position to examine the stereospecificity of the conversion of the appropriate inhibitors into pyruvate by D-amino acid aminotransferase and by L-aspartate aminotransferase. (2*R*,3*S*)-[3-$^2H_1$]- and (2*R*,3*R*)-[2,3-$^2H_2$]-serine *O*-sulfates, **2b** (R = SO_3H, H_B = ^2H) and **2b** (R = SO_3H, H_A = ^2H), respectively, and (2*S*,3*S*)-β-chloro-[3-$^2H_1$]- and (2*S*,3*R*)-β-chloro-[2,3-$^2H_2$]-alanines, **1b** (H_B = ^2H) and **1b** (H_A = ^2H), respectively, were therefore incubated separately with D-amino acid aminotransferase in the presence of lactate dehydrogenase, NADH and ^3H_2O. Protonation of the putative

aminoacrylate intermediate **11** would yield [3-$^2H_1$,3-$^3H_1$]pyruvate and it was hoped that immediate reduction to L-lactate **15** by lactate dehydrogenease would prevent any possible racemisation/exchange α- to the carbonyl group of pyruvate. The samples of lactate were purified using semi-preparative HPLC on an RP-C_{18} column. They were converted into acetate **16** either by chromic acid oxidation[17] or by Lemieux oxidation[18] at elevated temperatures. The samples of acetate were assayed for their configurational composition by conversion into malate (hydroxybutanedioate) and use of fumarase in the usual manner.[19] The *F*-values and enantiomeric excesses (ee) are shown in Table 1, experiments 1–4, and indicate that the turnover of both suicide substrates involves stereoselective protonation of the equivalent face of the aminoacrylate intermediate during the process. The stereoselectivity indicates that the process involves retention of stereochemistry for the elimination/proton addition involved in the overall reaction

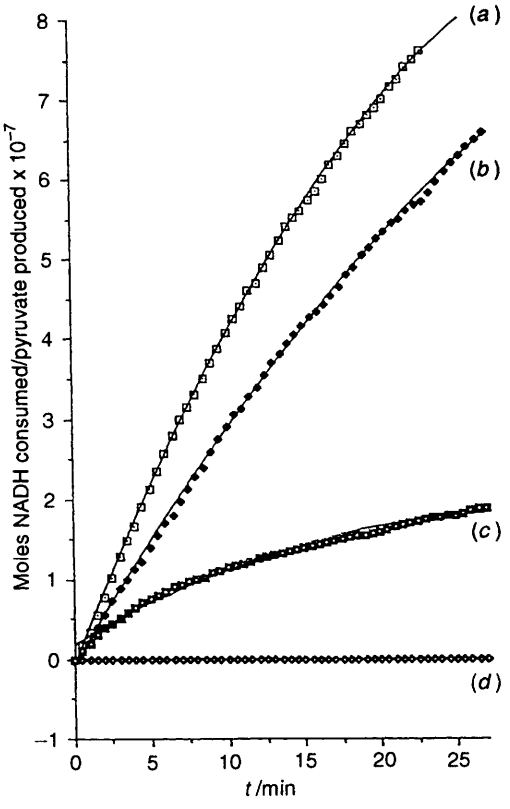


Fig. 1 (a) Incubation of D-amino acid aminotransferase with β-chloro-D-alanine **1b** in the presence of lactate dehydrogenase and NADH; (b) as for (a) but with D-serine *O*-sulfate **2b** as substrate; (c) incubation of L-aspartate aminotransferase with L-serine *O*-sulfate **2a** in the presence of lactate dehydrogenase and NADH; (d) as for (c) but using L-alanine aminotransferase instead of L-aspartate aminotransferase. Concentrations were identical in all four experiments.

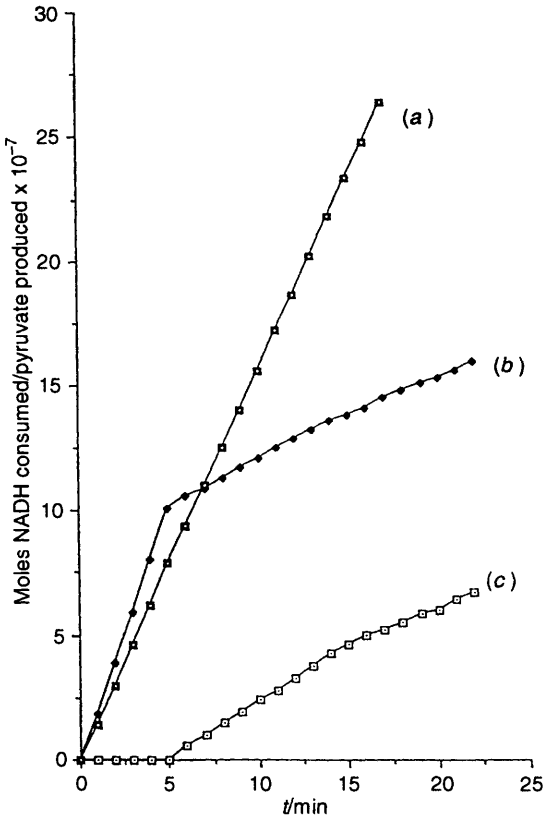


Fig. 2 (a) Incubation of L-alanine aminotransferase with L-alanine and α-ketoglutarate in the presence of lactate dehydrogenase and NADH; (b) as for (a) but with L-serine *O*-sulfate added after 5 min (3.6 mmol) and 12 min (5.4 mmol); (c) incubation of L-alanine aminotransferase with L-serine *O*-sulfate in the presence of lactate dehydrogenase and NADH with α-ketoglutarate added after 3 min and L-alanine after 5 min

Table 1 Stereochemical analysis of the samples of lactate obtained on incubation of (2*R*,3*S*)-[3-$^2H_1$]serine *O*-sulfate, (2*R*,3*R*)-[2,3-$^2H_2$]serine *O*-sulfate, (2*S*,3*S*)-β-chloro[3-$^2H_1$]alanine and (2*S*,3*R*)-β-chloro-[2,3-$^2H_2$]alanine with D-amino acid aminotransferase (EC 2.6.1.21) in the presence of lactate dehydrogrenase (EC 1.1.1.27) and NADH, and of (2*S*,3*R*)-[3-$^2H_1$]serine *O*-sulfate and (2*S*,3*S*)-[2,3-$^2H_2$]serine *O*-sulfate with L-aspartate aminotransferase (EC 2.6.1.1) in the presence of lactate dehydrogenase and NADH

Experiment	Enzyme	Substrate	*F*-value[a] (CrO_3 oxidation)	ee	*F*-value[a] (Lemieux degradation)	ee
1	D-Amino acid aminotransferase (EC 2.6.1.21)	(2*R*,3*S*)-[3-$^2H_1$]serine *O*-sulfate	53.5	12% *R*	55.6	19% *R*
2	D-Amino acid aminotransferase	(2*R*,3*R*)-[2,3-$^2H_2$]serine *O*-sulfate	45.1	17% *S*	38.1	41% *S*
3	D-Amino acid aminotransferase	(2*S*,3*S*)-β-chloro-[3-$^2H_1$]alanine	54.3	15% *R*		
4	D-Amino acid aminotransferase	(2*S*,3*R*)-β-chloro-[2,3-$^2H_2$]alanine	47.1	10% *S*	47.4	9% *S*
5	L-Aspartate aminotransferase (EC 2.6.1.1)	(2*S*,3*R*)-[3-$^2H_1$]serine *O*-sulfate	51.3	*RS*	50.2	*RS*
6	L-Aspartate aminotransferase	(2*S*,3*S*)-[2,3-$^2H_2$]serine *O*-sulfate	50.3	*RS*	49.8	*RS*

[a] The *F*-value,[20] the percentage tritium retention in the fumarase reaction, is related to the enantiomeric excess (ee) by equation (1).

$$ee = \frac{|50 - F|}{29} \times 100\% \qquad (1)$$

$F > 50 \equiv$ excess of *R* enantiomer; $F < 50 \equiv$ excess of *S* enantiomer.

from **1b** or **2b** to pyruvate **8** *via* steps **1** (or **2**) ⟶ **4** ⟶ **5** ⟶ **6** ⟶ **9** ⟶ **11** ⟶ **14** ⟶ **8** of Schemes 1–3.

The values are consistent for both substrates and are of the same order as those found earlier when stereospecifically tritiated samples of D- and L-serine *O*-acetate were incubated with an amino acid racemase in ^2H_2O, where again overall retention of stereochemistry was indicated.[21]

We then incubated (2*S*,3*R*)-[3-$^2H_1$]- and (2*S*,3*S*)-[2,3-$^2H_2$]-serine *O*-sulfates, **2a** (R = SO_3H, H_B = ^2H) and **2a** (R = SO_3H, H_A = ^2H), respectively with L-aspartate aminotransferase in the presence of lactate dehydrogenase, NADH and ^3H_2O. The purified samples of pyruvate were then oxidised to acetate and assayed for chirality as before. The results (shown in Table 1, experiments 5 and 6) indicated that, within the limits of accuracy of the method, the samples were racemic.

Although racemisation during degradation of lactate had not previously been a problem,[19] we conducted the degradation and assay using a method developed by Cornforth to minimise racemisation.[22] The results still indicated that, within the limits of error, the samples were racemic.

We have thus shown that, whereas the overall elimination/protonation reaction shows modest stereoselectivity with overall retention when either β-chloro-D-alanine **1b** or D-serine *O*-sulfate **2b**, R = SO_3H is used as substrate with D-amino acid aminotransferase, the corresponding reaction of L-serine *O*-sulfate **2a**, R = SO_3H catalysed by L-asparate aminotransferase shows no stereoselectivity. This may indicate that protonation of the aminoacrylate **11** occurs partially within and partially outside the active site of the former enzyme, whereas protonation occurs entirely outside the active site of the latter enzyme.

Experimental

M.p.s were determined on a Kofler hot-stage apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer PE241 polarimeter using a 1 dm path-length micro cell; $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. IR spectra were recorded on a Perkin-Elmer 1720 Fourier transform instrument and UV spectra were recorded on a Philips PU 8720 spectrophotometer. Mass spectra were recorded by Mr. A. Greenway using Kratos MS25 and Kratos MS80 instruments and on KS50 and VG7070 instruments by Dr. S. Chotai at the Wellcome Research Laboratories, Beckenham. All ^1H NMR spectra were recorded on a Bruker WM360 instrument (360 MHz); *J*-values are given in Hz, and the residual solvent peak was used as reference.

(2R)-*Serine* O-*Sulfate Hydrochloride* **2b** (R = SO_3H).—(2*R*)-Serine **2b** (R = H) (1.0 g, 9.52 mmol), was dissolved in vigorously stirred trifluoroacetic acid (TFA) (10 cm^3) at room temperature under nitrogen. Chlorosulfonic acid (0.95 cm^3, 14.3 mmol) was added dropwise to the vigorously stirred mixture, which was left at room temperature for 20 min. Ethanol (1 cm^3) was added to destroy excess of chlorosulfonic acid and diethyl ether (100 cm^3) was added. The solid product was collected by centrifugation and was recrystallised from aq. acetone. The crystals were collected by filtration, washed with ice-cold diethyl ether and dried (1.13 g, 54%); m.p. 220–223 °C (lit.,[12] 230 °C for L-isomer); $[\alpha]_D^{22.5}$ −9.84 (*c* 3.4, 1 mol dm^{-3} HCl) (lit.,[12] +9.8 for L-isomer); *m/z* (+ve FAB, glycerol) 186 ([M + H]$^+$); ν_{max}(KBr)/cm^{-1} 3421 (NH), 3200–2500 (CO*OH*) and 1765 (C=O); δ_H[$(CD_3)_2SO$] 4.08 (1 H, **ABX**, $J_{3R,3S}$ 11.3, $J_{3R,2}$ 4.3, 3-H_R), 4.14 (1 H, **ABX**, $J_{3R,3S}$ 11.3, $J_{3S,2}$ 2.8, 3-H_S) and 4.20 (1 H, br, 2-H).

(2*R*,3*S*)-[3-$^2H_1$]Serine *O*-sulfate hydrochloride, **2b** (R = SO_3H, H_B = ^2H), was prepared as above in 40% yield using (2*R*,3*S*)-[3-$^2H_1$]serine **2b** (R = H, H_B = ^2H);[10] m.p. 222–225 °C; *m/z* (+ve FAB, glycerol) 187 ([M + H]$^+$); ν_{max}(KBr)/ cm^{-1} 3434 (NH), 3200–2500 (CO*OH*) and 1765 (C=O); δ_H[$(CD_3)_2SO$] 4.08 (1 H, d, $J_{3R,2}$ 4.8, 3-H_R) and 4.19 (1 H, br, 2-H).

(2*R*,3*R*)-[2,3-$^2H_2$]Serine *O*-sulfate hydrochloride, **2b** (R = SO_3H, H_A = ^2H), was prepared as above in 40% yield using (2*R*,3*R*)-[2,3-$^2H_2$]serine **2b** (R = H, H_A = ^2H);[10] m.p. 223–226 °C; *m/z* (FAB, glycerol) 188 ([M + H]$^+$; ν_{max}(KBr)/cm^{-1} 3429 (NH), 3200–2500 (CO*OH*) and 1763 (C=O); δ_H[$(CD_3)_2SO$] 4.12 (1 H, s, 3-H_S).

(2*S*)-Serine *O*-sulfate hydrochloride, **2a** (R = SO_3H) was prepared as above in 58% yield using (2*S*)-serine **2a**, R = H; m.p. 223.5–225 °C (lit.,[12] 230 °C); $[\alpha]_D^{22.5}$ +9.85 (*c* 3.4, 1 mol dm^{-3} HCl); *m/z* (+ve FAB, glycerol) 186 ([M + H]$^+$); ν_{max}(KBr)/cm^{-1} 3420 (NH), 3200–2500 (CO*OH*) and 1763 (C=O); δ_H[$(CD_3)_2SO$] 4.08 (1 H, **ABX**, $J_{3S,3R}$ 11.4, $J_{3S,2}$ 4.3, 3-H_S), 4.14 (1 H, **ABX**, $J_{3S,3R}$ 11.4, $J_{3R,2}$ 2.4, 3-H_R) and 4.20 (1 H, br, 2-H).

(2*S*,3*R*)-[3-$^2H_1$]Serine *O*-sulfate hydrochloride, **2a** (R = SO_3H, H_B = ^2H), was prepared as above in 53% yield using (2*S*,3*R*)-[3-$^2H_1$]serine **2a** (R = H, H_B = ^2H);[11] m.p. 222–225 °C; *m/z* (+ve FAB, glycerol) 187 ([M + H]$^+$); ν_{max}(KBr)/cm^{-1} 3441 (NH), 3200–2500 (CO*OH*) and 1765 (C=O); δ_H[$(CD_3)_2SO$] 4.08 (1 H, d, $J_{3S,2}$ 4.5, 3-H_S) and 4.19 (1 H, t, *J* 4.5, 2-H).

(2*S*,3*S*)-[2,3-$^2H_2$]Serine *O*-sulfate hydrochloride, **2a** (R = SO_3H, H_A = ^2H), was prepared as above in 58% yield using (2*S*,3*S*)-[2,3-$^2H_2$]serine **2a** (R = H, H_A = ^2H);[11] m.p. 222–225 °C; *m/z* (+ve FAB, glycerol) 188 ([M + H]$^+$); ν_{max}(KBr)/ cm^{-1} 3414 (NH), 3200–2500 (CO*OH*) and 1763 (C=O); δ_H[$(CD_3)_2SO$] 4.11 (1 H, s, 3-H_R).

Purification of D-*Amino Acid Aminotransferase*.—This was achieved by the following combination and adaptation of the methods of K. Soda *et al.*[15] and W. M. Jones *et al.*[16] *B. sphaericus* was grown in an aqueous medium (200 dm^3) composed of tryptone (2400 g), yeast extract (4800 g), glycerol (800 cm^3), K_2HPO_4 (2508 g) and KH_2PO_4 (462 g). The growth was carried out at 30 °C for 18 h under aeration and agitation in a 200 dm^3 fermentation vessel. The harvested cells were stored frozen in liquid nitrogen. *All subsequent operations were performed as close to* 0 °C *as possible.* All buffers used contained 0.1 mmol dm^{-3} dithiothreitol.

Step 1. *Breakage of cells.* Cells (1.5 kg) were defrosted and suspended in 50 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2 (1500 cm^3). The cells were ruptured by pressing the suspension twice through a Manton Gaulin homogeniser and intact cells and cell debris were removed by centrifugation (10 000 rpm) for 1 h. Salicylaldehyde assay[16] indicated that the supernatant contained 9.3 × 10^3 units of enzyme activity and protein (58.5 g) was shown to be present by the method of Warburg and Christian.[23]

Step 2. *Treatment with protamine sulfate.* Protamine sulfate (11.7 g, 20 mg/100 mg of protein) was added to the stirred supernatant. After 30 min the nucleic acid precipitate was removed by centrifugation (10 000 rpm) for 30 min. Assay showed the presence of 9.2 × 10^3 units of enzyme activity and protein (57.9 g) in the supernatant.

Step 3. *Fractionation with* 30% *aq. ammonium sulfate.* The supernatant was brought to 30% saturation (w/v) with solid $(NH_4)_2SO_4$ and the mixture was stirred for 30 min. The precipitate was removed by centrifugation (10 000 rpm) for 30 min. 8.5 × 10^3 Units of enzyme activity and protein (30.3 g) were shown to be present in the supernatant.

Step 4. *Fractionation with* 60% *aq. ammonium sulfate and dialysis.* Solid $(NH_4)_2SO_4$ was added to the supernatant to effect 60% saturation (w/v) and the mixture was stirred for 30 min. The precipitate was collected by centrifugation

(10 000 rpm) for 40 min. The pellet was dissolved in a minimal volume of 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2, to give a final total volume of 420 cm^3. This was dialysed (cellulose tubing) for 12 h against several changes of the same buffer. There were 8.4×10^3 units of enzyme activity and protein (25.4 g) after this step.

Step 5. *DE52 Cellulose column.* The clear enzyme solution was applied to a 7 × 28 cm column of DE52 (Whatman) cellulose equilibrated with the same buffer. The enzyme was eluted using a gradient formed with 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2 (500 cm^3), in the mixing chamber and 200 mmol dm^{-3} K_2HPO_4 buffer, pH 8.4 (500 cm^3). The fractions containing activity were pooled. A considerable loss of activity resulted from this step, leaving a total activity of 1.45×10^3 units and protein (3.74 g).

Step 6. *Fractionation with* 60% *aq. ammonium sulfate and dialysis.* The pooled column fractions were brought to 60% saturation (w/v) with solid $(NH_4)_2SO_4$ and the mixture was stirred for 30 min. The precipitate was collected by centrifugation (10 000 rpm) for 30 min and the pellet was dissolved in a minimal volume of 10 mol dm^{-3} K_2HPO_4 buffer, pH 8.2, to give a final volume of 90 cm^3. This was dialysed (cellulose tubing) for 8 h against several changes of the same buffer. Assay showed the presence of 1.01×10^3 units of enzyme activity and protein (277 mg).

Step 7. *Sephadex G-150 column.* The clear enzyme solution was applied to a 4 × 30 cm column of Sephadex G-150 equilibrated with 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2. It was eluted with the same buffer, the fractions containing activity being pooled. There were now only 198 units of enzyme activity and protein (48 mg). The enzyme could be stored frozen for several months without a decrease in its activity.

Comparison of the Rates of Conversion of β-*Chloro*-D-*alanine and* D- *or* L-*Serine* O-*Sulfate with* D-*Amino Acid Aminotransferase,* L-*Aspartate Aminotransferase and* L-*Alanine Aminotransferase.*—(1) D-*Amino acid aminotransferase.* D-Amino acid aminotransferase (2 units) in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2, was incubated at 30 °C separately with (2*S*)-β-chloroalanine hydrochloride (0.72 mg, 4.5 μmol) and (2*R*)-serine *O*-sulfate hydrochloride (1.0 mg, 4.5 μmol) in the presence of lactate dehydrogenase (70 units) and NADH (1.78 mg, 2.5 μmol) in a total volume of 2 cm^3. The rate of production of pyruvate was measured spectrophotometrically by following the decrease in absorption at 340 nm in the UV spectrum due to the oxidation of NADH to NAD^+, catalysed by lactate dehydrogenase. Further NADH was added every few minutes to replace that which had been consumed. The results are plotted as curves (a) and (b) respectively in Fig. 1.

(2) L-*Aspartate aminotransferase and* L-*alanine aminotransferase.* L-Aspartate aminotransferase (2 units) and L-alanine aminotransferase (2 units) in 10 mmol dm^{-3} K_2HPO_4, pH 8.2, were each incubated separately at 30 °C with (2*S*)-serine *O*-sulfate hydrochloride (1.0 mg, 4.5 μmol) in the presence of lactate dehydrogenase (70 units) and NADH (1.78 mg, 2.5 μmol) in a total volume of 2 cm^3. Further NADH was added as needed and the progress of the incubation was monitored by following the decrease in absorption at 340 nm in the UV spectrum. The results are plotted as curves (c) and (d) respectively in Fig. 1. In each case, a control incubation was carried out in which the enzyme under investigation was replaced by 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2.

Investigation of Inhibition of L-*Alanine Aminotransferase by* L-*Serine* O-*Sulfate.*—(1) *Incubation with normal substrates.* L-Alanine aminotransferase (2.5 units) in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2, was incubated at 30 °C with L-alanine (1.0 mg, 11 μmol) and α-ketoglutarate (2.0 mg, 13.7 μmol) in the presence of lactate dehydrogenase (70 units) and NADH (1.78 mg, 2.5 μmol) in a total volume of 2.5 cm^3. The progress of the reaction was followed by observing the decrease in absorption at 340 nm in the UV spectrum due to the oxidation of NADH to NAD^+. Further NADH was added when necessary to replace that which had been consumed. Results are shown as curve (a) of Fig. 2.

(2) *Incubation with substrate followed by addition of inhibitor.* The procedure followed for incubation (1) was repeated. After 5 min, (2*S*)-serine *O*-sulfate hydrochloride (2.0 mg, 9.0 μmol) was added, followed by further (2*S*)-serine *O*-sulfate hydrochloride (1.0 mg, 4.5 μmol) after a further 7 min. The absorption at 340 nm in the UV spectrum was observed throughout and the consumed NADH was replaced as required. Results are shown as curve (b) Fig. 2.

(3) *Incubation with inhibitor followed by addition of substrates.* L-Alanine aminotransferase (2.5 units) in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2, was incubated at 30 °C with (2*S*)-serine *O*-sulfate hydrochloride (2.0 mg, 9.0 μmol) in the presence of lactate dehydrogenase (70 units) and NADH (1.78 mg, 2.5 μmol) in a total volume of 2.5 cm^3. After 3 min, α-ketoglutarate (2.0 mg, 13.7 μmol) was added. After a further 2 min, L-alanine (1.0 mg, 11 μmol) was added. Further (2*S*)-serine *O*-sulfate hydrochloride (1.0 mg, 4.5 μmol) was added after a further 9 min. The oxidation of NADH was observed in the UV spectrum and the NADH was replenished when necessary. Results are shown as curve (c) in Fig. 2.

Incubation of the Stereospecifically Deuteriated Samples of β-*Chloro*-D-*alanine and* D-*Serine* O-*Sulfate with* D-*Amino Acid Aminotransferase in* ^3H_2O.—D-Amino acid aminotransferase (10 units) in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 8.2 (10 cm^3), was mixed with ^3H_2O (0.2 cm^3, ~18 GBq) and left in a refrigerator for 2 h. The enzyme solution was divided into four portions (2.5 units each) and incubated at 30 °C with (a) (2*S*,3*S*)-β-chloro-[3-$^2H_1$]alanine hydrochloride **1b** (H_B = ^2H)[10] (3.0 mg, 18.5 μmol), (b) (2*S*,3*R*)-β-chloro-[2,3-$^2H_2$]-alanine hydrochloride **1b** (H_A = ^2H)[10] (3.0 mg, 18.5 μmol), (c) (2*R*,3*S*)-[3-$^2H_1$]-serine *O*-sulfate hydrochloride **2b** (R = SO_3H, H_B = ^2H) (3.0 mg, 13.5 μmol), (d) (2*R*,3*R*)-[2,3-^2H]serine *O*-sulfate hydrochloride **2b** (R = SO_3H, H_A = ^2H) (3.0 mg, 13.5 μmol), each in the presence of lactate dehydrogenase (Sigma, 70 units) and NADH (3.56 mg, 5.0 μmol). The decrease in absorption at 340 nm in the UV spectrum was monitored, and when no further NADH was seen to be consumed a few drops of aq. trichloroacetic acid were added to each incubation mixture to precipitate the protein. The precipitate was removed by centrifugation (10 000 rpm) for 1 h and the pellet was washed with water (0.5 cm^3) and centrifuged (10 000 rpm) for 1 h. The two supernatant solutions thus produced were combined and the solvent was removed by trap-to-trap transfer. The four crude lactate samples were purified using a semi-preparative HPLC RP-C_{18} column with 20% methanol/80% 1 mmol dm^{-3} HCl, pH 2.7, as the mobile phase. The solvent was removed from each of the four pure lactate samples by trap-to-trap transfer to yield the products as solids.

Incubation of (2S,3R)-[3-$^2H_1$]*Serine* O-*Sulfate* **2a** (R = SO_3H, H_B = ^2H) *with* L-*Aspartate Aminotransferase and Lactate Dehydrogenase in* ^3H_2O.—A solution (220 mm^3) of L-aspartate aminotransferase (Sigma, 94 units) in ^3H_2O (18 GBq) and K_2HPO_4 (0.35 mg) was added to 10 mmol dm^{-3} phosphate buffer, pH 7.5 (1.5 cm^3), with lactate dehydrogenase (Sigma, 84 units in 10 mm^3) and (2*S*,3*R*)-[3-$^2H_1$]serine *O*-sulfate **2a** (R = SO_3H, H_B = ^2H) (3.5 mg, 16 μmol) in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 7.5 (100 mm^3), in a UV cuvette at 30 °C. Batches (40 mm^3) of a solution of NADH [33.5 mg in 10

mmol dm^{-3} K_2HPO_4 buffer. pH 7.5 (350 mm^3)] were added as required as the absorbance at 340 nm decreased. After 5 h, NADH (160 mm^3) had been added and the mixture was left at 30 °C for a further 5 h. TFA (50 mm^3) was added and the solution was filtered through a microfilter and injected on to a semipreparative HPLC column RP C_{18}. Elution with 1 mmol dm^{-3} aq. HCl–MeOH (4:1) yielded a sample containing 486 KBq of specific radiativity, 7.6 MBq $mmol^{-1}$.

Incubation of (2S,3S)-[2,3-$^2H_2$]*Serine* O-*sulfate* **2a** (R = SO_3H, H_A = ^2H) *with* L-*Aspartate Aminotransferase and Lactate Dehydrogenase in* ^3H_2O.—This was conducted as above using substrate (3.7 mg) and adding NADH [15.8 mg in 10 mmol dm^{-3} K_2HPO_4 buffer, pH 7.5 (200 mm^3)] over a period of 4 h, after which time the reaction mixture was left for a further 8 h. The radioactivity of the final product was 1.23 MBq with a specific radioactivity of 9.5 MBq $mmol.^{-1}$

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